Characterization of *Escherichia coli* ExbD protein modification *in vivo*

Aruna Kumar^{1,2} and Kathleen Postle^{1,3,#}

RUNNING TITLE:

ExbD modification

KEYWORDS:

Escherichia coli, ExbD, TonB, ExbB, chemical modification, 2D gel electrophoresis

¹ School of Molecular Biosciences, Washington State University Pullman, WA 99164

² Present address: Amity Institute of Biotechnology, Amity University Uttar Pradesh, Sector - 125, Noida - 201 303 (UP)

³ Present address: Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802

For correspondence: E-mail kup14@psu.edu

ABSTRACT: The TonB system of *Escherichia coli* couples the protonmotive force of the cytoplasmic membrane to active transport of nutrients across the outer membrane. In the cytoplasmic membrane, this system consists of three known proteins, TonB, ExbB, and ExbD. ExbB and ExbD appear to harvest the protonmotive force and transmit it to TonB, which then makes direct physical contact with TonB-dependent active transport proteins in the outer membrane. Using two-dimensional gel electrophoresis, we found that ExbD exists as two different species with the same apparent molecular mass but with different pIs. The more basic ExbD species was consistently present, while the more acidic species arose when cells were starved for iron by the addition of iron chelators. The cause of the modification was, however, more complex than simple iron starvation. Absence of either TonB or ExbB protein also gave rise to modified ExbD under iron-replete conditions where the wild-type strain exhibited no ExbD modification. The effect of the *tonB* or *exbB* mutations were not entirely due to iron limitation since an equally iron-limited *aroB* mutation did not replicate the ExbD modification. This constitutes the first report of *in vivo* modification for any of the TonB system proteins.

For Gram-negative bacteria, the characteristic outer membrane (OM) poses a dilemma. It protects the cells from environmental assailants such as detergents and antibiotics, but it limits the nutrients that can be obtained by diffusion to those ~600 da or less (1). Transport of larger, scarce or very important nutrients is accomplished by a wide variety of active transport proteins in the OM that obtain the energy from the protonmotive force of the cytoplasmic membrane (CM) (2-8). Energy transduction proceeds cyclically through a complex of integral CM proteins ExbB, ExbD, and TonB, with ExbB/D harvesting the protonmotive force and transducing it to TonB protein, which then transmits the energy for active transport to a TonB-dependent transporter (TBDT) in the OM (9-11). The TBDTs consist of a 22-stranded β -barrel that is entirely occluded by an amino terminal globular domain, called the cork (12). At least one role of TonB is to energize movement of the cork to allow transport into the periplasm (13-17).

Although involved in transport of many different nutrients such as nickel, sugar, and vitamin B12, for *Escherichia coli* the main function of the TonB system is to acquire the normally biologically unavailable element iron (18, 19). In an aerobic environment at neutral pH, iron is present in virtually insoluble ferric-hydroxide complexes. Likewise, mammalian hosts keep levels of soluble iron low by the presence of iron-binding proteins such as transferrin and lactoferrin (20). To acquire iron the bacteria therefore synthesize and secrete molecules with high affinities for iron (21). These siderophores (*Gr.* iron bearer) can chelate and solubilize the iron, with the iron-siderophore complex subsequently transported across the OM, energized by the TonB system. Expression of TonB, and the unlinked operon encoding ExbB, and ExbD are regulated ~ 3-to-4-fold under aerobic conditions by the availability of iron and the Fur repressor protein (9, 22, 23). Under iron limiting conditions, the expression of TonB, and potentially therefore also ExbD, is induced as the culture grows to stationary phase due to progressive iron

starvation (24). The expression of FepA, the OM transporter of the only siderophore synthesized by domesticated *E. coli* K12, is regulated over a wider range of ~ 30-fold (9). The *E. coli* K12 siderophore, enterochelin, is synthesized from chorismate as an offshoot of the pathway for aromatic amino acid biosynthesis by a non-ribosomal peptide synthetase (25). Both enterochelin (also known as enterobactin) and its biosynthetic intermediates are absent in *aroB* strains (26).

The integral cytoplasmic membrane proteins TonB, ExbB, and ExbD appear to form a complex in the cytoplasmic membrane with TonB extending across the periplasm to mechanically energize active transport through customized beta barrels in the outer membrane (11, 17, 27-29). TonB and ExbD are anchored in the cytoplasmic membrane by their N-termini, with the majority of each protein extending into the periplasmic space (Fig. 1) (30-32). With ExbB acting as a scaffold, the relationship between TonB and ExbD periplasmic domains proceeds through a four-stage cycle where the protonmotive force of the cytoplasmic membrane is required for ExbD to configure TonB for correct interaction with TonB-dependent transporters (TBDTs) (11, 17).

To understand more about the role of ExbD in the energy transduction process and search for possible chemical modifications, we evaluated its behavior by 2-dimensional (2D) gel electrophoresis. Membrane protein preparations were made from cells grown as liquid cultures in minimal M9 medium supplemented with additional aromatic amino acids as described previously (13) to mid or late exponential phase and pelleted by centrifugation. The pellet was suspended in 10mM HEPES buffer at 4° C and lysed by French pressure cell 3 times at 20000 psi. All subsequent manipulations took place at 4° C until samples were prepared for gel analysis. Unlysed cells were removed by centrifugation at 1000x g. Membranes were pelleted at

40 K rpm (~140,000 x g) for 2hr. The pellet was solubilised in 1.5ml of 10mM HEPES, 0.5% Na-deoxycholic acid with shaking for 2hr. The unsolubilised material was pelleted at 29 K (~75,000 x g) for 1hr. Solubilised membrane proteins were analyzed on conventional 2-dimensional gels following BIO-RAD's protocol for mini-PROTEAN II, 2D Cell. In the first dimension, ampholines in the pH range of 3-10 were used. The second-dimension gel was 13% SDS polyacrylamide (Fig 2). ExbD was detected on the 2D separation at pI range of 4.5-5.5 by immunoblot with α -ExbD polyclonal antibodies (Fig 2c), consistent with the calculated pI of ExbD at 4.7. Our initial experiment examined over-expressed ExbD from KP1038 [GM1, *exbB::Tn10, tolQ_{am}*, (33)] carrying plasmid pKP390 (*exbB/D* operon under arabinose control in pBAD24) induced with 0.2% arabinose, where two different spots with the same molecular mass but different apparent pI values were detected (Table 1, Fig. 2).

Observations on overexpressed ExbD could be subject to artifact. To determine the cause of the ExbD modification, we then completed all further studies in a strain background where ExbD was expressed in its normal chromosomal context in GM1 (*ara*, Δ *lac-pro*, *thi*; F' *pro*, *lac*), referred to in this paper as "wild-type". Bacterial strains were grown in M9 medium supplemented with additional aromatic amino acids as described (13) to either mid-exponential or late exponential phase under a variety of iron availabilities (Fig. 3). In the presence of added iron ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ at 1.85 μM or 100 μM), only one form of ExbD was evident (rows a, b; columns D, E). However, in the absence of any added iron, an apparently modified, more acidic, version of ExbD appeared when cells were grown to late exponential phase (compare column C in rows a and b) and were expected to be more iron-starved (24). The intensity of the modified more acidic ExbD spot increased as the cells were starved for iron by growth in the presence of iron chelators 2,2'-dipyridyl (DIP) at 200 μM and diethylenetriaminepentaacetic acid (DTPA) at

100 μ M (Fig. 3, rows a and b, columns A and B). Thus, the degree of ExbD modification was greater when the cells were starved for iron. It was not possible to grow the strains that are intrinsically iron-limited (*aroB*, *fepA*, *tonB*, *tonB* + *fepA*, or *exbB*) in the presence of iron chelators.

Fur is a global repressor of siderophore transport genes and enterochelin biosynthetic genes, as well as *tonB*, *exbB* and *exbD* (23, 24, 34). The effect of a Fur mutation (strain KP1422; GM1, *fur*::Tn5), which can mimic the transcriptional regulatory effects of iron limitation on the TonB system (9), was determined for the ExbD modification for cells grown with high levels of iron and iron starved cells (+DIP). The finding of only one major form of ExbD in the presence of 100 μ M Fe and two equally abundant forms of ExbD in the presence of DIP suggested that the effect on ExbD modification occurred downstream of its transcriptional regulation by Fur (Fig. 3, row c, columns B, E). These results further suggested that the protein which modified ExbD might not be regulated by Fur.

In vivo interaction of TonB with TBDT FepA is enhanced in presence of its transport ligands (17). In addition, TonB undergoes a conformational change following contact with a TBDT in the presence of enterochelin that does not occur in its absence (35). To test whether the absence of enterochelin (the sole TonB-dependent ligand synthesized by *E. coli* K12) affected ExbD modification, ExbD encoded by strain KP1274 (GM1, *aroB*, *zhf-50*::Tn10) was evaluated on immunoblots of 2D gels (Fig. 3, rows d,e; columns C, D, E). Similar results were seen for parental strain GM1 at both mid- and late-exponential phase, which ruled out the possibility that enterochelin-dependent signaling through FepA was the source of the modification. Similar results were also obtained for the strain KP1487 lacking FepA (W3110, Δ *fepA*::*kan*; Fig 3, row f).

There is evidence for signal transduction among TonB, ExbB, and ExbD in the cytoplasmic membrane. *In vivo*, ExbB and ExbD proteins are needed to generate conformational changes in the TonB C-terminus and for TonB to respond conformationally to the presence or absence of protonmotive force (10, 35, 36). The TonB N-terminal transmembrane domain is required for correct configuration of its C-terminus for subsequent interaction with ExbD (11). An intrinsically disordered region near the N-terminal transmembrane domain of ExbD regulates interaction of its C-terminus with TonB (37). To study effects of the absence of TonB, the modification of ExbD was examined in KP1484 (GM1, $\Delta tonB::kan$) and KP1491 (W3110, $\Delta tonB::kan$, $\Delta fepA$) (Fig. 3, rows g, h). In both cases, when TonB was absent a greater degree of ExbD modification was detected in columns C and D compared to wild-type bacteria grown under the same conditions. The $\Delta fepA$ mutation did not detectably change the degree of ExbD modification seen in row h.

Strains lacking *tonB* or the ability to synthesize enterochelin (*aroB*) are intrinsically iron-limited. The increase in ExbD modification seen with the *tonB* mutants could therefore be simply due to differences in degree of iron limitation. To indirectly compare steady state iron levels among wild-type, *aroB*, and *tonB* strains, levels of OM protein FepA, which is iron regulated (9, 38), were assessed in the media with various degrees of iron availability (Fig. 4). Strains were grown in M9 media with different levels of iron or iron chelators and harvested for immunoblot analysis by precipitation with trichloro-acetic acid (TCA) at $A_{550} = 0.5$. Harvesting with TCA prevents degradation by endogenous proteases that can occur even during boiling in gel sample buffer (39). While the level of FepA expression decreased in wild-type cells with the addition of increasingly greater levels of iron as seen previously (9), the levels of FepA in *aroB* or *tonB* cells remained constant and were essentially identical in all growth media. Although the

tonB and *aroB* mutations led to similar degrees of iron limitation, only the *tonB* mutation led to increased ExbD modification (Fig. 3, in columns C and, especially D, compare rows d, f with g, h). The same result was observed for cells harvested at $A_{550} = 0.85$ (data not shown).

To determine the level of ExbD modification in the absence of ExbB, strain KP1038 (GM1 *exbB::Tn10, tolQ*) was used with plasmid pKP378 expressing ExbD under control of the arabinose promoter to compensate for the polarity of the *Tn10* insertion in *exbB* on *exbD* expression (23). In M9 medium with ampicillin (100 $\mu\text{g/ml}$) and glycerol as carbon source, 0.008% arabinose induced ExbD expression to levels that mimicked those of a chromosomally encoded *exbD* gene. In the absence of iron or in 1.85 μM Fe, strains grew slowly and were harvested at $A_{550} = 0.64$ or 0.68 respectively. Similar to the ΔtonB strains, the absence of ExbB resulted in significantly higher levels of modified ExbD in both the absence of any iron and in 1.85 μM iron (Fig. 3, row i, columns C, D). Wild-type strains grown to $A_{550} = 0.5$ or 0.8 (Fig. 3, rows a, b columns C, D) did not exhibit this increased ExbD modification, thus ruling out growth-phase as a relevant variable. Thus, it appeared that the increased modification could be due to changes that occurred within the TonB/ExbB/ExbD complex during the energy transduction cycle.

We determined if agents known to disrupt the TonB system changed the degree of ExbD modification. The TonB complex responds conformationally to the presence or absence of protonmotive force (10, 11, 35). To determine if collapse of the protonmotive force played a role in ExbD modification, the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added at 50 μM to GM1 cells at $A_{550} = 0.85$ for 5 min at 37 $^{\circ}\text{C}$ with shaking and the cells were processed for the 2D gel analysis of ExbD. 50 μM CCCP completely and immediately stops bacterial growth and TonB system activity (40). There was no change compared to wild-

type cells in iron-limited media or iron-replete media (Fig. 3, compare row j, columns B, E with row a, columns B, E). Therefore, the ExbD modification appeared to occur even in the absence of TonB system function, as it also did in the absence of TonB and ExbB.

In the absence of continued protein synthesis, all the proteins in the TonB system have half-lives of ~ 120 min, while TonB activity as measured by various indirect assays decays with a half-life of ~ 20 min (11, 33, 41). This disparity suggests the presence of additional, as yet unknown, proteins in the TonB system since protein synthesis inhibitors have no effect on protonmotive force (Skare and Postle, unpublished observation). To determine if the effect of protein synthesis inhibition on ExbD modification, GM1 cells were grown in M9 medium to $A_{550} = 0.7$. Spectinomycin was added at 200 $\mu\text{g/ml}$ and the cells were incubated for 1 hour at 37 °C (final $A_{550} = 0.79$). There was no significant change in the extent of ExbD modification compared to wild-type cells in iron-limited media or iron-replete media (Fig. 3, row k, columns B, E; compare to row b, columns A, B, E).

It seemed likely that the modification of ExbD was occurring through its periplasmic domain, since there are only ~ 20 residues predicted to occupy the cytoplasm and those could be sequestered by ExbB [Fig. 1; (11)]. Phosphorylation of periplasmic proteins has been documented (42), however, in that case it did not appear to be of functional significance (43). Neither ExbD nor its modified form could be detected by immunoblots with anti-phosphoserine, anti-phosphotyrosine, or anti-phosphothreonine antibodies (data not shown), suggesting that the modification was either not phosphorylation or that it did not occur through those residues.

To the best of our knowledge, this is a first report of a chemical modification of any component of the TonB system. We have not determined the role it plays, however we thought

it was sufficiently important to future research to publish the data so that others can take note of them.

Acknowledgements

We thank Charles Bulathsinghala for construction of KP1484 and Surendran Devanathan for construction of KP1487 and KP1491. We thank Michael Konkel for instruction in the 2D gel electrophoresis technique. We thank Anne Ollis and Ray Larsen for critical reading of the manuscript. We gratefully acknowledge the support of the National Institute of General Medical Sciences through grant GM42146 to K.P.

References

1. **Nikaido H.** 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* **67**:593-656.
2. **Hancock REW, Braun V.** 1976. Nature of the energy requirement for the irreversible adsorption of bacteriophages T1 and $\phi 80$ to *Escherichia coli*. *J. Bacteriol.* **125**:409-415.
3. **Schalk IJ, Abdallah MA, Pattus F.** 2002. A new mechanism for membrane iron transport in *Pseudomonas aeruginosa*. *Biochem Soc Trans* **30**:702-705.
4. **Klebba PE.** 2003. Three paradoxes of ferric enterobactin uptake. *Front Biosci* **8**:s1422-1436.
5. **Wandersman C, Delepelaire P.** 2004. Bacterial iron sources: from siderophores to hemophores. *Annu Rev Microbiol* **58**:611-647.
6. **Buchanan SK.** 2005. Bacterial metal detectors. *Mol Microbiol* **58**:1205-1209.
7. **Braun V.** 2006. Energy transfer between biological membranes. *ACS Chem Biol* **1**:352-354.
8. **Postle K, Larsen RA.** 2007. TonB-dependent energy transduction between outer and cytoplasmic membranes. *Biometals* **20**:453-465.
9. **Higgs PI, Larsen RA, Postle K.** 2002. Quantitation of known components of the *Escherichia coli* TonB-dependent energy transduction system: TonB, ExbB, ExbD, and FepA. *Mol. Microbiol.* **44**:271-281.
10. **Ollis AA, Postle K.** 2012. ExbD mutants define initial stages in TonB energization. *J Mol Biol* **415**:237-247.
11. **Gresock MG, Kastead KA, Postle K.** 2015. From homodimer to heterodimer and back: Elucidating the TonB energy transduction cycle. *J Bacteriol* **197**:3433-3445.
12. **Noinaj N, Guillier M, Barnard TJ, Buchanan SK.** 2010. TonB-dependent transporters: regulation, structure, and function. *Annu Rev Microbiol* **64**:43-60.
13. **Devanathan S, Postle K.** 2007. Studies on colicin B translocation: FepA is gated by TonB. *Mol Microbiol* **65**:441-453.
14. **Ma L, Kaserer W, Annamalai R, Scott DC, Jin B, Jiang X, Xiao Q, Maymani H, Massis LM, Ferreira LC, Newton SM, Klebba PE.** 2007. Evidence of ball-and-chain transport of ferric enterobactin through FepA. *J Biol Chem* **282**:397-406.
15. **Hickman SJ, Cooper REM, Bellucci L, Paci E, Brockwell DJ.** 2017. Gating of TonB-dependent transporters by substrate-specific forced remodelling. *Nat Commun* **8**:14804.
16. **White P, Joshi A, Rassam P, Housden NG, Kaminska R, Goult JD, Redfield C, McCaughey LC, Walker D, Mohammed S, Kleanthous C.** 2017. Exploitation of an iron transporter for bacterial protein antibiotic import. *Proc Natl Acad Sci U S A* **114**:12051-12056.
17. **Gresock MG, Postle K.** 2017. Going outside the TonB Box: Identification of novel FepA-TonB interactions in vivo. *J Bacteriol* **199**.

- 225 18. **Braun V.** 2003. Iron uptake by *Escherichia coli*. Front Biosci **8**:s1409-1421.
- 226 19. **Schauer K, Rodionov DA, de Reuse H.** 2008. New substrates for TonB-dependent
227 transport: do we only see the 'tip of the iceberg'? Trends Biochem Sci **33**:330-338.
- 228 20. **Sheldon JR, Laakso HA, Heinrichs DE.** 2016. Iron acquisition strategies of bacterial
229 pathogens. Microbiol Spectr **4**.
- 230 21. **Winkelmann G.** 2002. Microbial siderophore-mediated transport. Biochem Soc Trans
231 **30**:691-696.
- 232 22. **Young GM, Postle K.** 1994. Repression of *tonB* transcription during anaerobic growth
233 requires Fur binding at the promoter and a second factor binding upstream. Molecular
234 Microbiology **11**:943-954.
- 235 23. **Ahmer BMM, Thomas MG, Larsen RA, Postle K.** 1995. Characterization of the
236 *exbBD* operon of *Escherichia coli* and the role of ExbB and ExbD in TonB function and
237 stability. J. Bacteriol. **177**:4742-4747.
- 238 24. **Postle K.** 1990. Aerobic regulation of the *Escherichia coli tonB* gene by changes in iron
239 availability and the *fur* locus. J. Bacteriol. **172**:2287-2293.
- 240 25. **Crosa JH, Walsh CT.** 2002. Genetics and assembly line enzymology of siderophore
241 biosynthesis in bacteria. Microbiol Mol Biol Rev **66**:223-249.
- 242 26. **Pittard AJ.** 1987. Biosynthesis of the Aromatic Amino Acids, p. 368-394. In Ingraham
243 JL, Low KB, Magasanik B, Schaechter M, Umberger HE (ed.), *Escherichia coli* and
244 *Salmonella Typhimurium*, Cellular and Molecular Biology. American Society for
245 Microbiology, Washington D.C.
- 246 27. **Braun V, Gaisser S, Herrmann C, Kampfenkel K, Killman H, Traub I.** 1996.
247 Energy-coupled transport across the outer membrane of *Escherichia coli*: ExbB binds
248 ExbD and TonB *in vitro*, and leucine 132 in the periplasmic region and aspartate 25 in
249 the transmembrane region are important for ExbD activity. J. Bacteriol. **178**:2836-2845.
- 250 28. **Larsen RA, Letain TE, Postle K.** 2003. *In vivo* evidence of TonB shuttling between the
251 cytoplasmic and outer membrane in *Escherichia coli*. Mol Microbiol **49**:211-218.
- 252 29. **Kaserer WA, Jiang X, Xiao Q, Scott DC, Bauler M, Copeland D, Newton SM,
253 Klebba PE.** 2008. Insight from TonB hybrid proteins into the mechanism of iron
254 transport through the outer membrane. J Bacteriol **190**:4001-4016.
- 255 30. **Roof SK, Allard JD, Bertrand KP, Postle K.** 1991. Analysis of *Escherichia coli* TonB
256 membrane topology by use of PhoA fusions. J. Bacteriol. **173**:5554-5557.
- 257 31. **Karlsson M, Hannavy K, Higgins CF.** 1993. A sequence-specific function for the N-
258 terminal signal-like sequence of the TonB protein. Mol. Microbiol. **8**:379-388.
- 259 32. **Kampfenkel K, Braun V.** 1992. Membrane topology of the *Escherichia coli* ExbD
260 protein. J. Bacteriol. **174**:5485-5487.
- 261 33. **Skare JT, Postle K.** 1991. Evidence for a TonB-dependent energy transduction complex
262 in *Escherichia coli*. Mol Microbiol **5**:2883-2890.

- 263 34. **Seo SW, Kim D, Latif H, O'Brien EJ, Szubin R, Palsson BO.** 2014. Deciphering Fur
264 transcriptional regulatory network highlights its complex role beyond iron metabolism in
265 *Escherichia coli*. *Nat Commun* **5**:4910.
- 266 35. **Larsen RA, Thomas MG, Postle K.** 1999. Protonmotive force, ExbB and ligand-bound
267 FepA drive conformational changes in TonB. *Mol. Microbiol.* **31**:1809-1824.
- 268 36. **Baker KR, Postle K.** 2013. Mutations in *Escherichia coli* ExbB transmembrane domains
269 identify scaffolding and signal transduction functions and exclude participation in a
270 proton pathway. *J Bacteriol* **195**:2898-2911.
- 271 37. **Kopp DR, Postle K.** 2020. The Intrinsically Disordered Region of ExbD is Required for
272 Signal Transduction. *J Bacteriol*.
- 273 38. **McIntosh MA, Earhart CF.** 1977. Coordinate regulation by iron of the synthesis of
274 phenolate compounds and three outer membrane proteins in *Escherichia coli*. *J.*
275 *Bacteriol.* **131**:331-339.
- 276 39. **Postle K.** 2007. TonB system, *in vivo* assays and characterization. *Methods in*
277 *Enzymology* **422**:245-269.
- 278 40. **Bulathsinghala CM, Jana B, Baker KR, Postle K.** 2013. ExbB cytoplasmic loop
279 deletions cause immediate, proton motive force-independent growth arrest. *J Bacteriol*
280 **195**:4580-4591.
- 281 41. **Kadner RJ, McElhaney G.** 1978. Outer membrane-dependent transport systems in
282 *Escherichia coli*: Turnover of TonB function. *J. Bacteriol.* **134**:1020-1029.
- 283 42. **Celis RT.** 1984. Phosphorylation in vivo and in vitro of the arginine-ornithine
284 periplasmic transport protein of *Escherichia coli*. *Eur J Biochem* **145**:403-411.
- 285 43. **Celis RT, Leadlay PF, Roy I, Hansen A.** 1998. Phosphorylation of the periplasmic
286 binding protein in two transport systems for arginine incorporation in *Escherichia coli* K-
287 12 is unrelated to the function of the transport system. *J Bacteriol* **180**:4828-4833.
- 288 44. **Sun TP, Webster RE.** 1986. *fli*, a bacterial locus required for filamentous phage
289 infection and its relation to colicin-tolerant *tolA* and *tolB*. *J. Bacteriol* **165**:107-115.
- 290 45. **Hayashi K, Morooka N, Yamamoto Y, Fujita K, Isono K, Choi S, Ohtsubo E, Baba**
291 **T, Wanner BL, Mori H, Horiuchi T.** 2006. Highly accurate genome sequences of
292 *Escherichia coli* K-12 strains MG1655 and W3110. *Mol Syst Biol* **2**:2006 0007.
- 293 46. **Skare JT, Ahmer BMM, Seachord CL, Darveau RP, Postle K.** 1993. Energy
294 transduction between membranes - TonB, a cytoplasmic membrane protein, can be
295 chemically cross-linked *in vivo* to the outer membrane receptor FepA. *J. Biol. Chem.*
296 **268**:16302-16308.
- 297 47. **Higgs PI, Letain TE, Merriam KK, Burke NS, Park H, Kang C, Postle K.** 2002.
298 TonB interacts with nonreceptor proteins in the outer membrane of *Escherichia coli*. *J*
299 *Bacteriol* **184**:1640-1648.
- 300 48. **Held KG, Postle K.** 2002. ExbB and ExbD do not function independently in TonB-
301 dependent energy transduction. *J Bacteriol* **184**:5170-5173.

49. **Koster W.** 2005. Cytoplasmic membrane iron permease systems in the bacterial cell envelope. *Front Biosci* **10**:462-477.

Table 1.

Bacterial strains and plasmids used

Strains or plasmids	Properties	Source or reference
GM1	<i>ara</i> (<i>pro-lac</i>) <i>thi</i> F' <i>pro Lac</i>	(44)
W3110	F- IN(<i>rrnD-rrnE</i>)I <i>rph-1</i>	(45)
KP1038	GM1 (<i>exbB</i> ::Tn10, <i>tolQ</i>)	(33)
KP1092	GM1(<i>fepA</i> :: <i>kan</i>)	(46)
KP1274	GM1 (<i>aroB zhf-50</i> ::Tn10)	(47)
KP1344	W3110 Δ (<i>tonB</i> , <i>P14</i>):: <i>blaM</i>)	(35)
KP1422	GM1(<i>fur</i> ::Tn5)	(9)
KP1484	GM1, Δ <i>tonB</i> :: <i>kan</i>	This study
KP1487	W3110, Δ <i>fepA</i> :: <i>kan</i>	This study
KP1491	W3110, Δ (<i>tonB</i> , <i>P14</i>):: <i>kan</i> , Δ <i>fepA</i>	This study
pKP378	<i>exbD</i> gene in pBAD24	(48)
pKP390	<i>exbB/D</i> operon in pBAD24	(48)

Fig. 1. The TonB system. The siderophore enterochelin, containing an iron atom (green triangle), binds to the TonB-dependent transporter, FepA with sub-nanomolar affinity (29). Integral cytoplasmic membrane proteins TonB, ExbB and ExbD harvest the protonmotive force of the cytoplasmic membrane and somehow transduce it to FepA, causing it to release the enterochelin in the direction of the periplasmic space. From that point it is picked up by a periplasmic binding protein and carried to a cytoplasmic membrane ABC transporter for transport into the cytoplasm (49). Numbers in circles represent the per-cell ratios of each protein in *E. coli* W3110 grown under iron limiting conditions. Under those conditions, TonB is present at ~ 1300 copies per cell (9). [Adapted from (11), which also contains a model for the dynamics of the TonB system *in vivo*.]

Fig. 2. Two-dimensional (2D) gel electrophoresis analysis of ExbD protein.

A) Distribution of silver-stained pI markers in descending order of molecular mass: hen egg white conalbumin (pI = 6.0, 6.3, 6.6, MW = 76,000), bovine serum albumin (pI = 5.4, 5.6, MW = 66,200), bovine muscle actin (pI = 5.0, 5.1, MW = 43,000), rabbit muscle GAPDH (pI = 8.3, 8.5, MW = 36,000), bovine carbonic anhydrase (pI = 5.9, 6.0 MW = 31,000), soybean trypsin inhibitor (pI = 4.5, MW = 21,500), equine myoglobin (pI = 7.0, MW = 17,500). The arrow represents the direction of isoelectric focusing from basic (B) to acidic (A).

B) 2D analysis of total membrane fraction (see text) from strain GM1 grown to $A_{550} = 0.85$ (measured using a Spectronic 20 with a 1.5 cm pathlength) electrophoresed on 2D gels, and stained with Coomassie brilliant Blue.

C) 2D analysis of ExbD protein. Immunoblot of membrane fraction separated by 2D gel electrophoresis and developed using anti-ExbD antibody (9) at 1:5000 and secondary antibody at 1:10000.

Fig. 3 Differential modification of ExbD protein detected by 2D gel electrophoresis. Regions containing monomer ExbD-specific bands arising from the immunoblots similar to those in Fig. 1C are shown. a) and b) Strain GM1 grown either to $A_{550} = 0.85$ or $A_{550} = 0.50$; c) KP1422 (GM1 *fur*::Tn5) grown to $A_{550} = 0.85$; d) and e) KP1274 (GM1, *aroB*, *zhf-50*::Tn10) grown to $A_{550} = 0.85$ or $A_{550} = 0.50$; f) KP1487 (W3110, Δ *fepA*::*kan*) grown to $A_{550} = 0.85$; g) KP1484 (GM1, Δ (*tonB*, *P14*)::*kan*) grown to $A_{550} = 0.85$; h) KP1491 (W3110, Δ (*tonB*, *P14*)::*kan*, Δ *fepA*) grown to $A_{550} = 0.85$; i) KP1038 GM1 (*exbB*: Tn10, *tolQ*) carrying pKP 378 (*exbD*) was grown in the presence of 0.008% arabinose only to $A_{550} = 0.64$; j) GM1 grown to $A_{550} = 0.85$ and treated with 10 μ M CCCP (carbonyl cyanide 3-chlorophenylhydrazone) for 5 min prior to analysis on 2D

gels; k) GM1 grown to $A_{550} = 0.79$ and treated with 200 μ M spectinomycin for 1 hr. prior to analysis on 2D gels. It is important to note that only relative levels of ExbD and modified ExbD are being compared within each panel. Comparison of levels between panels is not shown. Arrow depicts direction of isoelectric focusing from basic to acidic.

A = 100 μ M diethylene- triamine –penta- acetic acid (DTPA); B = 200 μ M 2,2' Dipyridyl (Dip); C= No supplement added; D= 1.85 μ M of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; and E = 100 μ M of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ represent increasing degrees of iron abundance. Note that different A_{550} values were reached prior to processing for Aa (0.73), Ci (0.64), and Di (0.68), likely due to iron limitation.

Fig. 4 Relative FepA levels in various media are consistent with expected degrees of iron limitation. Strains were grown in minimal medium supplemented as in Fig. 3 to $A_{550} = 0.5$, harvested by precipitation TCA. Samples were electrophoresed on 11% SDS polyacrylamide gels and analyzed by immunoblot using anti-FepA primary antibody [1:5000; (9)] and secondary HRPO conjugated sheep/Goat anti- rabbit (1: 10000). WT= GM1; mut stands for *aroB* =KP1274 or *tonB* = KP1344.